A NEW MUTATION CAUSING MALE-MALE COURTSHIP IN *Drosophila*

A Senior Scholars Thesis

by

STACEY LARAE HANLON

Submitted to the Office of Undergraduate Research
Texas A&M University
In partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

May 2007

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Approved by:

Research Advisor: Ginger E. Carney
Associate Dean for Undergraduate Research: Robert C. Webb

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ABSTRACT

A New Mutation Causing Male-Male Courtship in *Drosophila* (May 2007)

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Courtship in *Drosophila* is an innate behavior, one that is hardwired into the genetic architecture of the fly. A small number of mutations are known to disrupt the genes controlling the neurological basis of courtship; even fewer have been documented which cause male flies to actively pursue and court other male flies. Two established examples of such genes are *fruitless* and *dissatisfaction*, which probably function in a small number of CNS neurons to regulate behavior. A strain of flies was identified from an EMS mutagenesis screen for which males homozygous for the mutation display a male-male courtship phenotype. Original data indicated the male-male courtship may be associated with a male-sterile mutation on the second chromosome, but when the hypothesis was first tested the results proved otherwise. In an effort to determine more about the nature and location of the male-male courtship mutation so that a mapping strategy could be developed, new mutant lines with isolated single chromosomes were established from the original strain. One subsequent line was found to exhibit vigorous male-male courtship and is currently being used to determine if now, when placed in the

known isogenized genetic background, the initial sterility mutation is still a player in producing the aberrant courtship.

DEDICATION

To anyone who needs a reminder that behavior is a phenotype.

ACKNOWLEDGEMENTS

I would like to thank the gracious guidance, patience, and pushing of Dr. Ginger Carney, my advisor, without whom I would surely not have developed the foundation required of an investigator.

I would also like to thank Lisa Ellis and Kara Boltz for their support and continued help in the lab. Their knowledge along with their friendship was of great importance in my success in this project.

Lastly, I would like to thank the Office of the Vice President of Research and the Office of Undergraduate Research which not only made the Undergraduate Research Scholars Program happen, but allowed it to flourish and assist in developing the minds of future scholars. Their funding and persistence have played a major role in the support of this project.

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CHAPTER I

INTRODUCTION

Why the fly?

For almost a century, geneticists have been utilizing the phenomenal genetic tool that is *Drosophila melanogaster*, otherwise known as the fruit fly. The father of fly genetics, Thomas Hunt Morgan, began using it as model in 1909, and it has since developed into one of the best understood eukaryotic organisms (SNUSTAD and SIMMONS 2000). The research of this one system has revealed a great deal of information in the areas of genetics, development, neurology, and biochemistry (just to name a few). What exactly makes *Drosophila* so enticing to work with? Though curly wings and rosy red eyes may be disputable assets as to why so many clamor to work with the fly, one can be assured that the ease of the system itself is most definitely a factor. Flies first begin as one of many eggs laid by a fertilized female. After about a day of development, the embryo hatches to reveal a wormlike larva that constantly eats and molts twice in order to increase in size. After five days, the larva turns its skin hard to form a pupa case for its metamorphosis. Four days later, after much tissue has degraded and formed, a fruit fly emerges that is now capable of flight and reproduction (SNUSTAD and SIMMONS 2000). At room temperature in a small container with a simple mixture of sugar and cornmeal for food, flies can propagate rather quickly and produce hundreds of progeny

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This thesis follows the style and format of GENETICS.

over a short time span. This attribute is beneficial not only for the continuation of stocks, but also essential for certain types of experiments including courtship assays where several flies of specific genotypes are collected on a daily basis.

Balancer Chromosomes: Powerful tools in Drosophila

Beyond the inexpensive upkeep and generally hassle-free husbandry of flies, there is still more to offer. The genome of the fly is bundled into only four chromosomes: one sex chromosome and three autosomes. The fourth chromosome is small and contains very little active genetic data, leaving the two larger autosomes to contain most of the genome. When attempting to map a mutation, if the trait is not sex-linked then the mutation will most likely fall onto either one of the larger autosomes rather than on the fourth chromosome. This small number of chromosomes allows one to perform genetic crosses with ease by limiting the possible number of variations among progeny, which in turn means more of the genotype needed to carry on experiments. To harness this advantage, H.J Muller formulated an idea for special chromosomes called "balancers" which most *Drosophila* research labs use daily (GREENSPAN 2004). Balancer chromosomes contain three important features: a special chromosomal arrangement of wild-type genes discouraging recombination during meiosis; a dominant marker gene; and a recessive lethal mutation (Figure 1). Normal chromosomes can pair up with balancers in the nucleus since there is enough homology between the two, but the overall inverted nature of the balancer's genetic material is effective in preventing homologous

recombination. This way, a recessive mutation associated with a particular chromosome can be followed through lineages without the risk of it being lost during meiosis. Detection of the balancer is done easily because of its built-in dominant marker—a gene producing a noticeable phenotypic trait. The marker provides a visual means for determining the genetic makeup of a fly, making the collection of a particular genotype quick and efficient. If, however, two similar balancers pair with one another during fertilization, the fly would not live to adulthood due to the recessive lethal mutation embedded in the balancer's genetic material. Thus, a fly homozygous for balancers on a chromosome cannot be mistaken for a fly heterozygous with the same balancer (GREENSPAN 2004).

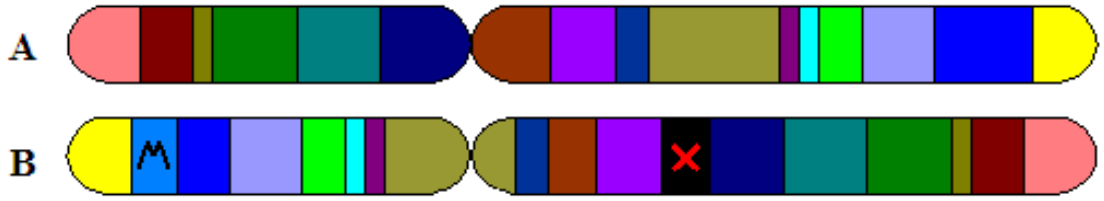


FIGURE 1.—A wild-type chromosome in comparison to a balancer chromosome. Though a balancer chromosome (B) is quite a bit different than a wild-type chromosome (A), it is all the same a powerful tool for use in *Drosophila* genetics. All variations of balancers contain a recessive lethal (denoted as an "X"), a dominant marker (denoted as an "M"), and slightly scrambled genetic material (shown by the mismatched assortment of colors). Though the wild-type copies of most genes are still provided by the balancer, they are out of conventional sequence and prevent recombination between regions of similar composition.

Finding a mutation: Deficiency Mapping

Balancers are great for keeping up with a mutation in a stock or following a chromosome through crosses, but when it comes to mapping the mutation, there are other useful tools

available. One such tool is the deficiency chromosome, or deletion chromosome, which is missing a small portion of its genetic material in a known location. When paired with another chromosome, only one copy of the deleted region will be present (as provided by the opposite chromosome), making the fly hemizygous for that portion of the genome (Figure 2).

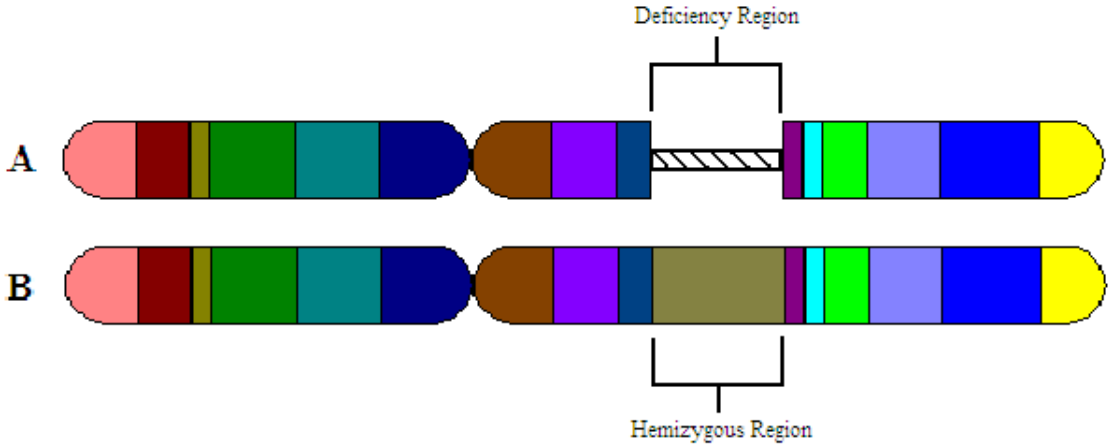


FIGURE 2.—How deficiency chromosomes work. A deficiency chromosome (A) lacks a portion of genetic material which allows its homologous chromosome (B) to be hemizygous for the corresponding region. Thus, if a recessive mutation is located in a region which is uncovered by a deficiency chromosome, then it will revealed in the phenotype.

Strains of flies missing specific regions of a single chromosome are for purchase from the Bloomington Stock Center; to map a mutation, a tiling strategy is put into action to walk through the chromosome one portion at a time. When a deficiency paired with the affected chromosome reproduces the phenotype seen in homozygous mutants, genes within the deletion region are studied to determine individual function and deduce which, when disrupted, is responsible for the mutant phenotype (GREENSPAN 2004).

Drosophila: a model organism for the study of behavior

In addition to its ease of use and array of available genetic tools, the fly is a formidable model organism for the study of behavior. One avenue of investigation is the genetic basis of reproductive behaviors. As seen in Figure 3, the elaborate courtship dance a male exhibits toward a female to ready her for copulation is an innate behavior. If a normal fertilized egg is isolated and allowed to develop into an adult, the fly will still partake in courtship with another of the opposite sex. No hints from other flies, no parental guidance, no examples to follow—amazingly the fly knows what to do without ever having seen another fly in its life. This phenomenon strongly suggests that courtship behavior is hardwired into the genetic material of *Drosophila* (BILLETER *et al.* 2002).

Courtship in *Drosophila melanogaster*

The dance a male performs while courting a female appears rather simplistic; a wing extension here, a leg tap there, and poof: the two copulate (Figure 3). The orchestrated moves are simple enough, but there is actually a lot more going on that cannot be seen. Besides visual stimuli, flies use gustatory, olfactory, and auditory cues to direct the progression of courtship.

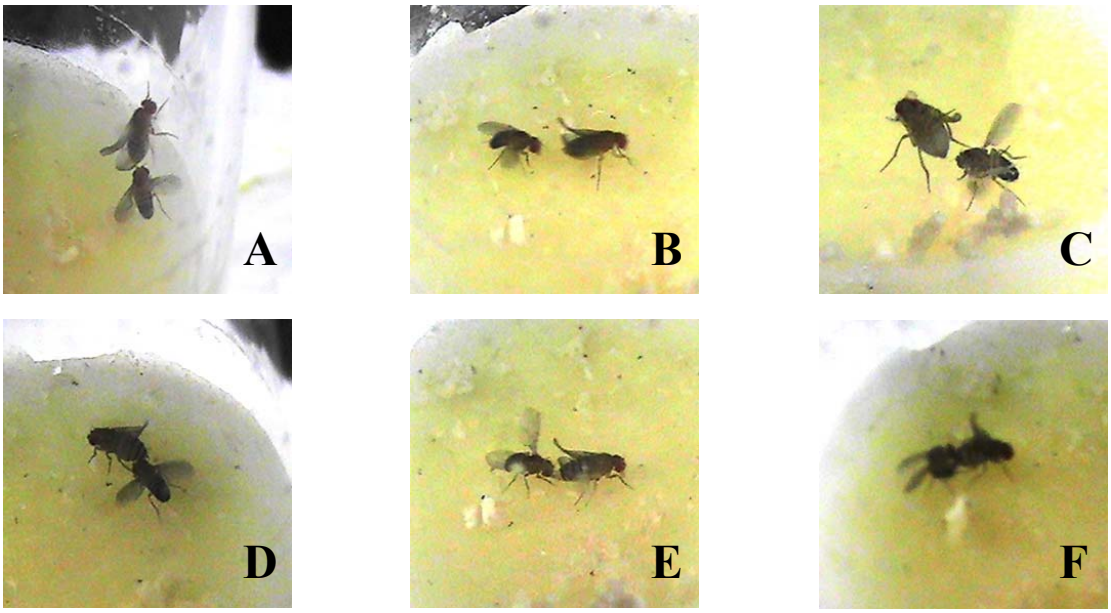


FIGURE 3.—Courtship in *Drosophila*. Above are pictures illustrating the basic courtship dance that is performed before copulation. First a male approaches a female and orients himself to initiate the courtship (A); when she begins to move he will follow (B) and "sing" to her through wing vibrations (C). He then collects various chemical cues by tapping her (D) in between pursuits and, if permitted, will lick her genitalia by extending his proboscis for even more cues (E). The male finishes by bending his abdomen to prepare for copulation (F); if she consents, they will mate for ~15-20 minutes.

Visual Cues

Before anything else, a male fly must be able to see the female. First he lands next to the female and positions himself to begin sending and receiving sensory signals. Some of the visual cues are dynamic and result in locomotor activity, the rest are fixed cues like shapes and contrast. As a receptive female begins to move, the male begins to follow behind her; this movement is an important visual cue for a male initiating courtship, and his ability to keep up with the female depends on the capability of his visual system (GREENSPAN and FERVEUR 2000). Throughout the pursuit, it is essential for the male to

correctly position himself in relation to the female to obtain further chemical cues and ensure subsequent copulation.

Pheromones

The pheromones produced by the female are readily taken up by the male through olfactory and gustatory sensory neurons. Though the majority of chemicals playing a role in courtship pass through contact between the flies, certain stimulatory pheromones are volatile and thus can be detected before any physical contact occurs (STURTEVANT 1915). *Drosophila* adults can produce anywhere from 12 to 17 different cuticular hydrocarbons, many of which are not extremely volatile and can only be detected in close (1-2mm) proximity (COBB and FERVEUR 1995). From the female's initial output of pheromones, a male can assess the potential success of his venture and either continues with courtship or turns away. The power of these chemicals is an important aspect in courtship—groups of males exposed to volatile female attractants will begin to display male-male courtship, and virgin females or recently fertilized females can emit inhibitory substances that discourage a male's pursuit (TOMPKINS *et al.* 1980).

Auditory Cues

A female fly can be easily swooned by the vibratory patterns contained in her mates' "love song"—females are stimulated by the song and tend to reduce their locomotion when exposed to it (VONSCHILCHER 1976). Furthermore, auditory cues allow the female to detect the species of her potential mate. From the patterns of humming, time intervals

between pulses, and rhythmic oscillation between the pulses, females can distinguish one *Drosophila* species from the next (RITCHIE *et al.* 1999).

The *Sex-lethal* pathway

Extensive research has shown courtship is part of a much bigger signaling system which is not only responsible for male and female behavior, but also controls the physical morphology of gender. The following is a general overview of this signaling cascade and its function in the fly (for an exhaustive review please see BAKER 1989; CLINE and MEYER 1996; PARKHURST and MENEELY 1994).

Sex determination in *Drosophila* begins with the ratio of the number of X chromosomes to the number of sets of autosomes in the fly, otherwise known as the X:A ratio. Normal males (XY) have a 1:2 ratio while normal females (XX) have a 2:2 ratio. When the ratio is greater than or equal to 1 (as in females), one of the first cellular responses is the activation of the *Sex-lethal* (*Sxl*) locus. This master regulator has two main functions, the first of which is to self-regulate its own expression (and thus giving each cell a "memory" of its sex), and the second is to turn on splicing regulators which will morphologically and behaviorally form a female. One of these regulators, *transformer* (*tra*) works in conjunction with *transformer-2* (*tra-2*) to regulate the splicing of downstream agents such as *fruitless* (*fru*) and *doublesex* (*dsx*). Though less is known about it, *dissatisfaction* (*dsf*) is also thought to be downstream of *tra* but independent of *fru* and *dsx,* making it a potential third branch in the sex-determination pathway. In

males, the *Sxl* pathway is not activated and there is no production of these splicing regulators, resulting in a different set of transcripts that is used for male somatic and nervous system development. Thus, male appears to be the default sex; if *Sxl* turns off in a female fly, *tra* cannot be activated and female transcripts cannot be produced, leaving the genetically female fly looking and acting like a male. Figure 4 provides a diagram of this sex-determination hierarchy.

Well Characterized mutants of the *Sxl* pathway

Since courtship is an innate behavior, it should be possible to modify it without completely disabling a nonsexual attribute. One strategy is to perform a screen and isolate hypomorphic mutants that display aberrant behavior. Hypomorphs help eliminate the possibility of isolating a non-neurological mutation that affects behavior since hypomorphic mutations are able to function somewhat normally in critical roles. The following is a summary of known genes under the control of *Sxl* and their neurological control of behavior in *Drosophila.*

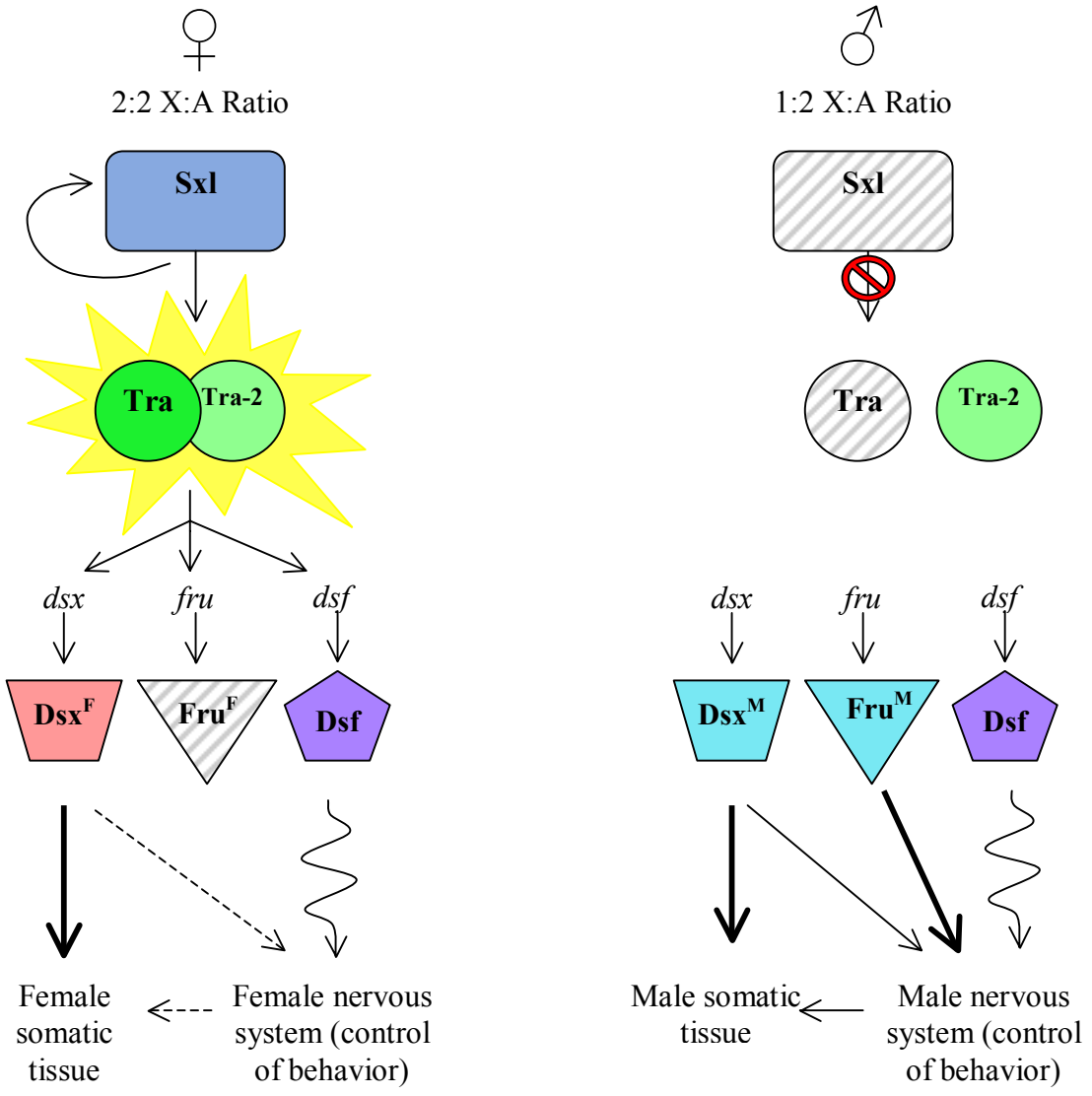
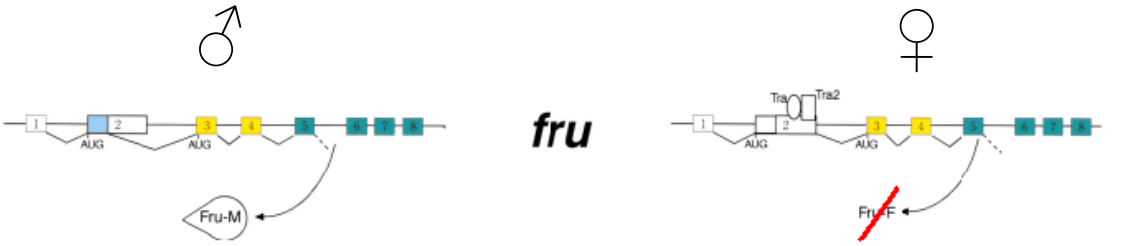


FIGURE 4.—The Sex Determination Pathway. This hierarchy determines maleness and femaleness at the genetic level in *Drosophila*. The master regulator, *Sxi*, is turned on in females and is responsible for activating several genes below it including *tra*. When activated, the Tra protein will pair with Tra-2 (active in both sexes) and regulate the splicing of transcription factors *dsx* and *fru*. Depending on the composition of the final transcript, either a male or female form of the protein will be made (i.e., Dsx^F and Dsx^M). Though it is primarily involved in somatic tissue formation, *dsx* has been shown to play a role in behavior; in males it is required for the formation of Fru^M-expressing neurons (BILLETER *et al.* 2006), and in females it can impact parts of the nervous system responsible for behaviors (hence the dashed arrow) (WATERBURY *et al.* 1999). The Fru^M protein is a main component in establishing behavior, but in females, the *fru* transcript is spliced and translated into a non-functional Fru^F protein (LEE *et al.* 2000). Recently, it has also been shown that *dsf* may be a third branch in the pathway. It is downstream of Tra (exactly how is yet to be determined as indicated by the curvy line) and plays a role in courtship behavior for both males and females in a *fru*- and *dsx*-independent manner (FINLEY *et al.* 1997).

The fruitless *gene*

First found during a sterility screen (GILL 1963), *fruitless* (*fru*) has been studied extensively for its role in controlling behavior (GAILEY and HALL 1989; MANOLI *et al.* 2005; VILLELLA *et al.* 1997). Roughly 140kb in length, it is controlled by four promoters (P1-P4) and encodes transcription factors with N-terminal Bric à brac, Tramtrack, and Broad-complex domains (BTB domains) and C-terminal Zinc finger motifs (RYNER *et al.* 1996). Sex-specific splicing of the P1-controlled transcript produces the male-specific protein Fru^M (expressed only in the nervous system) and is responsible for male behavior; females lack this P1-regulated protein product (LEE *et al.* 2000), but still produce non-sex-specific Fru proteins under the control of the other promoters (Figure 5) (ANAND *et al.* 2001). When Fru^M is expressed in females, the females exhibit male courtship behavior (MANOLI *et al.* 2005).



(Yamamoto et al. 1998)

FIGURE 5.—Schematic of the *fru* transcript. In females, Tra is present and works in conjunction with Tra-2 to effectively splice the *fru* transcript and produce a truncated non-functional product. In males, Tra is not made and thus the splicing pattern is different, leading to a functional male isoform of Fru (Fru^M).

This finding strongly supports the hypothesis that *fru* is sufficient for the performance of courtship. When Fru^M expression was visualized in the brain of fly, it was discovered to

be in several places, including primary sensory neurons and third-order neurons (MANOLI *et al.* 2005). The circuitry formed by the widespread locations of Fru^M allows for several sensory cues to be given, received, and reacted to, each of which is essential for successful execution of courtship. When *fru* is disabled in male flies, courtship is no longer only directed at females but begins to be toward males as well (GAILEY and HALL 1989). Different mutant alleles of *fru* produce similar phenotypes, such as the fru^{sat} mutant which prefers to only court males (ITO *et al.* 1996). Male-male courtship usually involves the close following, or chaining, of males so that something resembling a conga line forms (Figure 6). A "Chaining Index" (ChI) is used to assay courtship of this nature, a percentage which measures the amount of time males are following one another in groups of 3 or more over the total amount of time in observation. One of the best chaining strains of *fru* mutants is the original mutant found by Gill (fru^1). Males homozygous for the mutation can produce ChIs around 75% (VILLELLA *et al.* 1997).

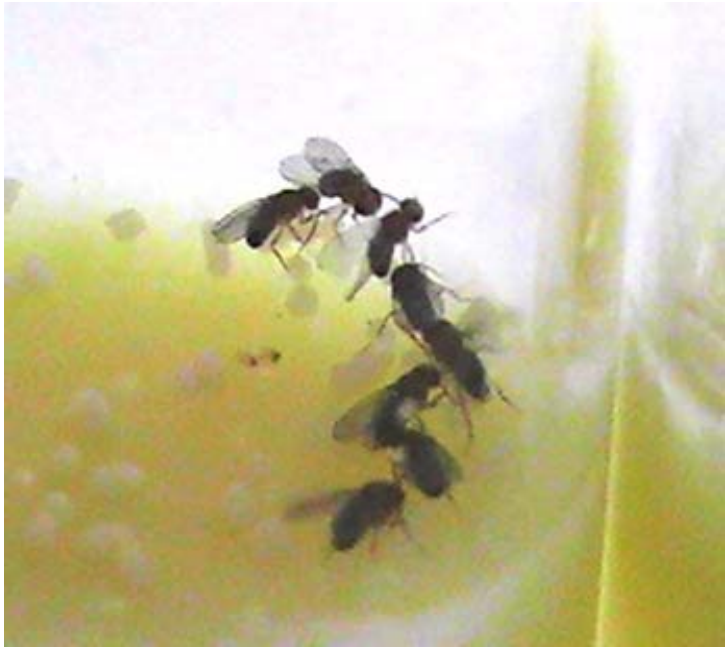


FIGURE 6.—Male-male chaining behavior. Males mutant for *fru* do not exclusively court females—they extend courtship to other males as well. When several of these mutants are placed together, they can form courtship "chains" as seen to the left. As the chain slinks around, the flies can produce other aspects of courtship such as wing extensions. The flies in this photo are homozygous for the mutant allele fru^{1} originally found by Gill in 1963.

The doublesex *gene*

On the other side of the coin is *doublesex* (*dsx*) which is responsible for the control of sex-specific morphology (BURTIS and BAKER 1989). Its transcripts are also sex-specifically spliced by Tra, resulting in functional male (Dsx^{M}) and female (Dsx^{F}) isoforms. These control the formation of sex-specific somatic and reproductive tissue (Figure 7) (BAKER and WOLFNER 1988; HOSHIJIMA *et al.* 1991).

FIGURE 7.— Schematic of the *dsx* transcript. The *dsx* transcript is similar to *fru* in its regulation; the presence of Tra will splice the transcript so that a female isoform of Dsx (Dsx^F) is made. When Tra is absent, normal splicing occurs producing the default male Dsx protein (Dsx^M).

In females, Dsx^F controls the formation of ovaries, genitalia, female pheromones, and abdominal pigmentation (BAKER and RIDGE 1980; MCROBERT and TOMPKINS 1985; WATERBURY *et al.* 1999). Conversely, male-specific tissue such as the gonads and sex combs are under the control of Dsx^M (JURSNICH and BURTIS 1993). Though females do not perform courtship when made to express Dsx^M, males lacking Dsx^M will generate courtship at diminished levels suggesting some form of *dsx* behavioral control (MCROBERT and TOMPKINS 1985; TAYLOR *et al.* 1994; VILLELLA and HALL 1996). Analysis of *dsx* expression reveal its presence in the brain, including in neurons near others expressing *fru*. This suggests it may play a neurological role in the control of behavior (LEE *et al.* 2002). If Dsx^F can repress formation of male-specific tissues and promote female morphology, and Dsx^M can repress formation of male-specific tissues and promote female morphology, then perhaps this same type of "switch system" is in use for behavior as well (Figure 8) (SHIRANGI *et al.* 2006). Activation of downstream targets of *fru* and *dsx* according to gender would be able to produce clear-cut on/off switches for behavior and morphology.

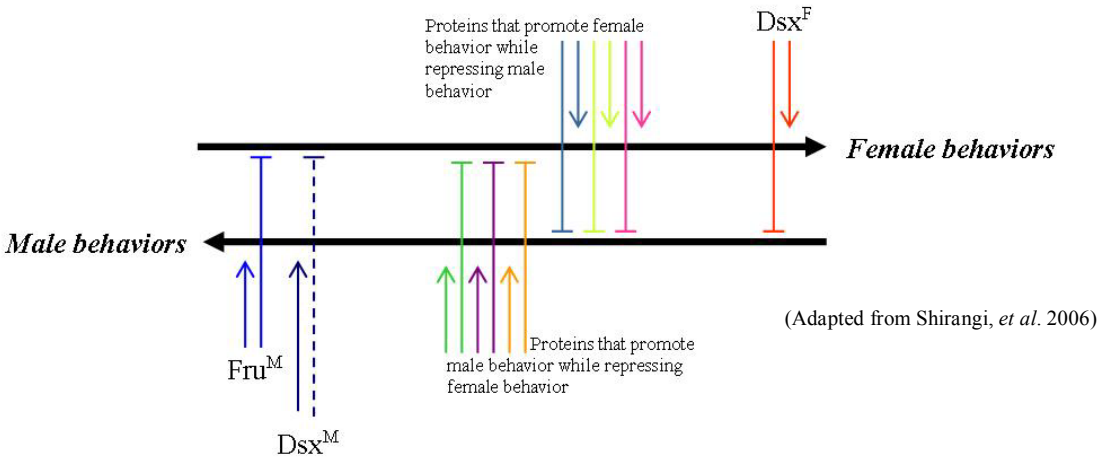


FIGURE 8.—A switch system responsible for the regulation of behavior. Though *fru* is the main determinant of behavior, it is possible that *dsx* may also be a contributor. With the help of other proteins acting as either "pro-female" or "pro-male," the behavioral and morphological aspects of gender can be established in the fly. These proteins represent targets of *fru* or *dsx* products or could be under the control of other unknown branches of the sex determination hierarchy. The dashed line indicates the unknown affects of Dsx^M on female behavior.

The dissatisfaction *gene*

Though not as much is known about the splicing and control of this gene, *dissatisfaction* (*dsf*) mutant males have abnormal behaviors similar to those of *fru* mutants including chaining and indiscriminant courtship (FINLEY *et al.* 1997; LEE *et al.* 2000). Not only are males abnormal, but mutant females reject courtship advances and fail to lay eggs. Studies show that *dsf* functions downstream of Tra and independently of *fru* and *dsx*, making it a candidate for a third branch of the sex determination hierarchy; however it has not been proven Tra directly acts on the transcript despite an intron resembling the Tra binding site (FINLEY *et al.* 1998). Thus, the normal function of the protein could be an example of a behavioral switch as modeled in Figure 8.

CHAPTER 2

IN THE SEARCH FOR A NEW MUTATION CAUSING MALE-MALE COURTSHIP

Several mutations have been studied which alter male perception, persistence, and performance of courtship in *Drosophila*, and the possibility of uncovering another is both important and exciting. By understanding the control of behavior in *Drosophila*, we can get closer to understanding the foundation of our behavior.

Preliminary Work

In an effort to disrupt nonessential genes controlling physiological functions, an ethyl methanesulfanate (EMS) mutagenic screen created strains of mutagenized flies altogether known as the Zuker Collection (KOUNDAKJIAN *et al.* 2004). Male flies were fed EMS and then mated to pass on any inheritable mutations in germ cells. One strain generated from this screen contained a recessive mutation on the second chromosome, referred to as 58-41 *cnbw*, causing male sterility and was maintained in stock by use of a balancer (termed as being "balanced"). Dr. Ginger Carney mapped the precise region of the sterility mutation using deficiency chromosomes and found it to be located in the center of the L arm around 35A (Figure 9).

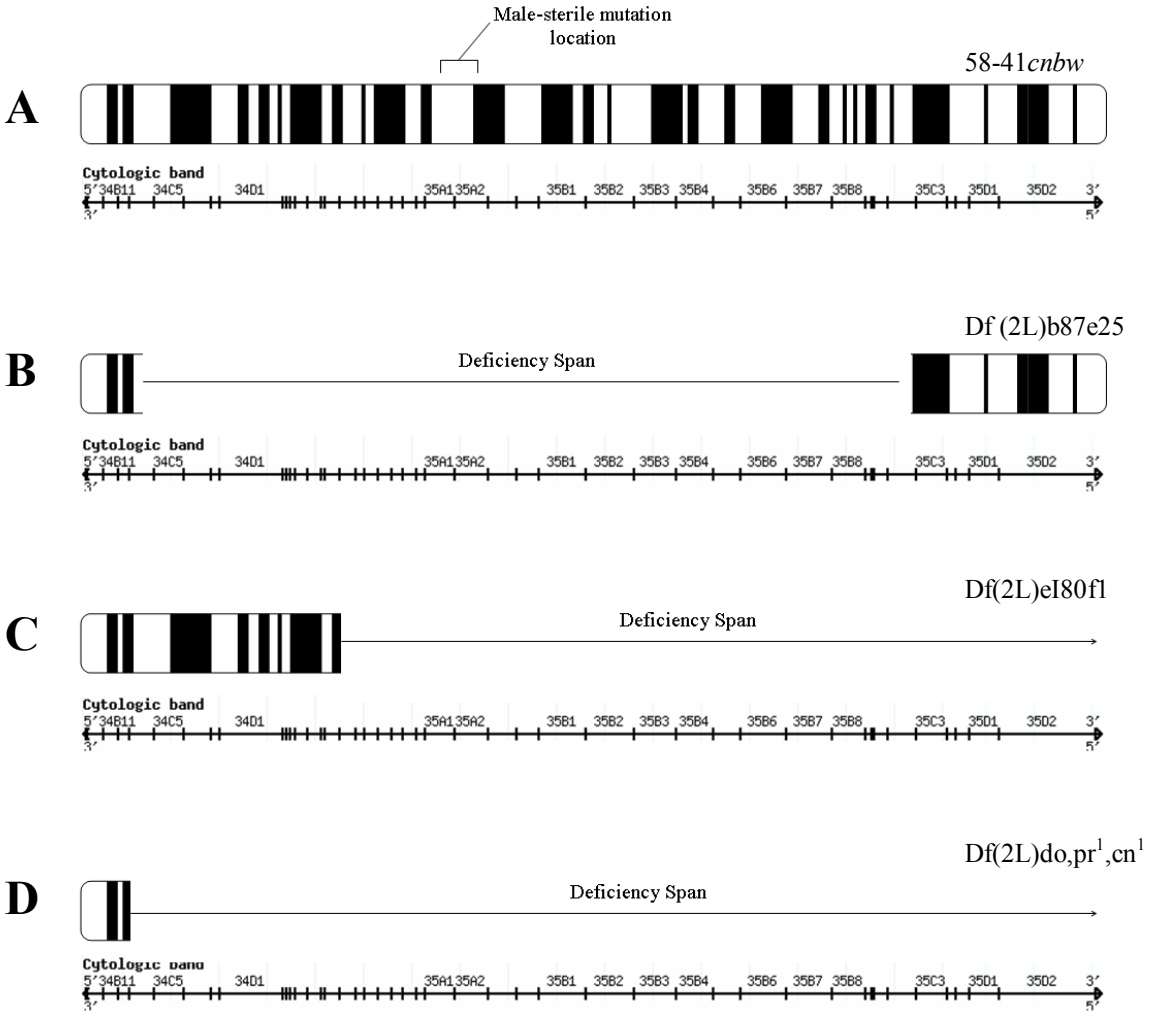


FIGURE 9.—Deficiency mapping results for the male sterility locus. In (A), the original 58-41*cnbw* chromosome is presented with the inferred location of the male sterility mutation. Initially, this mutation was thought to cause the male-male courtship behavior as well. Illustrated in (B), (C), and (D) with their respective deleted regions are the three deficiency chromosomes known to uncover the male-sterile mutation. These were later tested with the 58-41A *cn* chromosome to determine if the same male sterility mutation may be contributing to the male-male courtship; unfortunately all test results turned out negative.

To remove the *bw* marker, 58-41 *cnbw* was outcrossed with flies of known genetic material resulting in four new variations of the original strain: 58-41A *cn*, 58-41E *cn*, 58-41G *cn*, and 58-41H *cn*. Stocks were kept at 25ºC on regular 12h light and dark cycles. Preliminary behavioral observations of males homozygous for 58-41E *cn*

revealed that, when grouped, the males would court one another—a phenotype left up to me to verify. From each of the five strains, newly eclosed naïve males which had never mated (herein referred to as virgin males) and were homozygous for the second chromosome were collected for testing. They were first kept in solitary confinement for 7-10 days and then placed together in groups of 10-15 males in a yeasted food vial. Behavior was monitored with particular attention paid to any male-male courtship interactions. The conclusion was that the strongest evidence for any male-male courtship was in males homozygous for 58-41A *cn*, which raised the question of whether or not the sterility mutation was also responsible for the abnormal behavior. We tested this by pairing the deficiency chromosomes against the 58-41A *cn* chromosome and assaying for male-male courtship. If there was male-male courtship, then the sterility mutation (or a gene in the region uncovered by the deficiencies) may be responsible for the abnormal behavior (Figure 9).

Of the three deficiencies that successfully uncovered the sterility mutation, none produced male-male courtship behavior when paired with the 58-41A *cn* chromosome (Table 1). Positive controls with 58-41A*cn* homozygotes tested positive for male-male courtship, indicating the sterility mutation was not responsible for the abnormal behavior.

Experimental Strategy 1

Since male-male courtship was still observed in flies homozygous for the second-chromosome, it was unclear as to exactly what was responsible for it. One possibility is that during the mutagenic screen, a multiple mutation event occurred on the second chromosome to produce two mutations on one chromosome—one responsible for the sterility and the other causing the male-male courtship. A plan to tile through the entire second chromosome using deficiency chromosomes to find the second mutation was ready to begin.

In a turn of events, male-male courtship was seen between two curly-winged flies in the stock bottle. The dominant marker, Curly, is located on the balancer chromosome which supplies a wild-type copy of every gene on the second chromosome (see Figure 1 for review). Thus, if a second mutation on the 58-41A *cn* chromosome is causing the abnormal behavior, it should not be able to reveal itself when a wild-type copy is paired with it. Upon further investigation, it was ascertained that male flies heterozygous for the second chromosome (the 58-41A *cn* chromosome paired with a balancer) showed subtle signs of male-male courtship. This observation would not rule out the possibility that the mutation was still on the second chromosome—it could be dominant which meant the original plan to deficiency map it would not be able to continue. In contrast to that possibility was the apparent decrease in male-male courtship observed in the homozygotes which were originally very active in their behavior. Another explanation is the presence of a genetic modifier on the third chromosome in addition to the mutation

on the second influencing the male-male courtship phenotype. If there is involvement of a strong enhancer, it may cause expression of the male-male courtship in 58-41A *cn* heterozygotes; if a suppressor was responsible, then it could have been enabling semi-normal function in homozygotes leading to suppressed male-male courtship. Both of these modifier types may be in the population with serious implication on behavioral phenotypes. Thus, the best strategy was to isogenize the line and rid the strain of modifiers suppressing male-male courtship.

Experimental Strategy 2

Since the third chromosome is now a possible player in producing the male-male courtship phenotype, the best strategy to employ is one which will enable us to control the fly's genotype to the fullest extent. In balancing the third chromosome, the genetic composition can be controlled (single common chromosome, minimal recombination, etc.). Using a special stock containing balancers and dominant markers (so that each chromosome can be phenotypically scored), we devised a simple cross scheme as shown in Figure 10 allowing for a single individual chromosome to be followed from the P generation into the F_1 generation. This tracking was done to derive the best combination of chromosomes to produce a strong male-male courtship phenotype.

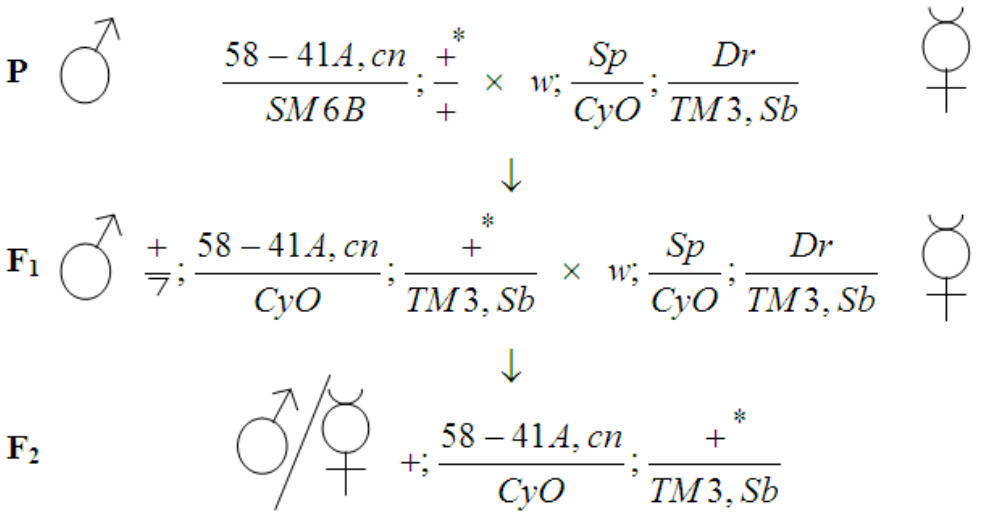


FIGURE 10.—Cross scheme implemented in Experimental Strategy 1. The cross scheme shown here balances the 58-41A *cn* strain for both the second and third chromosomes. A single male is crossed with a virgin female from the balancer strain and all F_1 male progeny are once again crossed with a virgin female from the initial balancer strain. Using this strategy, if one of the third chromosomes contain a courtship-enhancing modifier (as denoted with the asterisk*), it can be isolated and propagated for stock in the F_2 generation. Subsequent substrains can then be tested for male-male courtship.

After implementation of the cross design, instead of being able to study just two genotypes across one chromosome:

$$\left[\frac{58-41A\,cn}{Cyo}\right] \qquad [58-41A\,cn]$$

we were able to test of four genotypes across two chromosomes:

$$\left[\frac{58-41A\,cn}{Cyo},\frac{+}{TM3,Sb}^{*}\right] \qquad \left[58\text{-}41A\,cn,\frac{+}{TM3,Sb}^{*}\right] \qquad \left[\frac{58-41A\,cn}{Cyo},+^{*}\right] \qquad \left[58\text{-}41A\,cn,+^{*}\right]$$

After the crosses, a total of 17 substrains were created and tests began immediately. We gathered and isolated virgin males of each of the four possible genotypes in their own vials for 7-10 days. After their isolation we combined and observed them over a period

of 14 days for signs of male-male courtship. Fortunately, the fifth substrain tested (herein known as the BB strain) displayed strong male-male courtship when homozygous for the second chromosome and immediately chosen as the stock to be used for the upcoming assays.

First, it was necessary to confirm the initial observations of male-male courtship in the BB strain. Males representing each genotype were once again collected, isolated for 7-10 days, then combined and observed for 14 days for male-male courtship. After at least 5 tests of each genotype, we determined males homozygous for the second chromosome and either homozygous or heterozygous for the third displayed strong male-male courtship—even stronger than the original 58-41A *cn* strain. This evidence suggests the isogenization scheme lost a suppressor or retained a modifier, either possibility of which was beneficial for the advancement of the project. It is possible the mutation located on the second chromosome interacts with the modifier on the third, and when only one copy of the modifier is present, male-male courtship can still occur (for a more complete proposition, please see "Hypothesis: Putting Names to Faces" in Chapter III for perspectives as to what may be taking place). Thus, is courtship greater in flies homozygous for the second and third chromosomes? To answer this question, we created an assay based on one used for the measurement of courtship interaction in *fru* mutants (VILLELLA *et al.* 1997). Virgin male flies were collected and placed into isolation to age for 4 days before they were combined and aged for an additional 5 days in groups of eight. On day 10, the assay began by observing the groups via video for ten

minute intervals ten times a day over a period of five days. Intervals were later viewed to calculate a ChI for each vial at every interval. If ChIs are highest in males homozygous for the second and third chromosomes, then we can suspect the isogenization retained an element which enhances the phenotype. If ChIs are similar for flies homozygous for the second chromosome regardless of the genotype of the third, then the isogenization may have lost a suppressor. This result could also suggest one copy of the third chromosome is sufficient in producing the phenotype, and that two copies reinforces the male-male courtship without making it stronger.

Based on preliminary observations made of the isogenized BB line, it appears that flies homozygous for the second chromosome produce large ChIs irrespective of the genotype of the third chromosome. In morning tests conducted at the beginning of the light cycle, ChIs measured 84% and 88% for males homozygous for the second and third chromosomes and males homozygous for just the second chromosome, respectively. These ChI values, compared to those from males homozygous for the third chromosome or heterozygous for both the second and third chromosomes (0% and 0%, respectively), strongly indicates the mutation is still located on the second chromosome and is no longer under the influence of a repressor that may have initially been located on the third chromosome. Based upon these results, the deficiencies need to be tested again in this new 58-41A *cn* background to ensure they do not uncover a mutation producing the male-male courtship.

Experimental Strategy 3

The initial sterility mutation may still be a player in the production of this male-male courtship. To test this, we devised a complex crossing scheme (Figure 11). From it, flies with the 58-41A *cn* chromosome paired with one of the deficiencies will be tested; the third chromosome will either be balanced or unbalanced (homozygous) since courtship assay ChIs did not depend on the genotype of the third chromosome. Aside from the deficiency, the BB strain supplies all other genetic material; if males display male-male courtship, then we can say the mutation responsible maps somewhere within the region uncovered by the deficiency or, quite possibly, to the original sterility mutation. Controls for the experiment are the four genotypes of the BB strain used for the courtship assays. Males homozygous for the second chromosome are the positive controls, and males heterozygous for the second chromosome are the negative controls. The assay itself will proceed exactly like the courtship assays in Experimental Strategy 2, following the same aging, grouping, and observation protocols.

If the deficiency pairings with 58-41A *cn* do not produce male-male courtship, then we assume the sterility mutation (and thus the entire uncovered region) is not responsible for the abnormal behavior. The plan of action after these results is to map the second chromosome mutation using a mapping kit and the BB stock. If the deficiency mapping does not uncover a mutation causing male-male courtship, then several other (but difficult) possibilities are still available. Perhaps it is more necessary than anticipated to have the third chromosome homozygous, thus a mapping strategy incorporating a

crossing technique similar to Figure 11 may be the solution. Another possibility is a dosage-depended mutation: due to the load of mutant gene product required to produce the male-male courtship phenotype, two mutant gene copies must be active (not just one, like when uncovered by a deficiency).

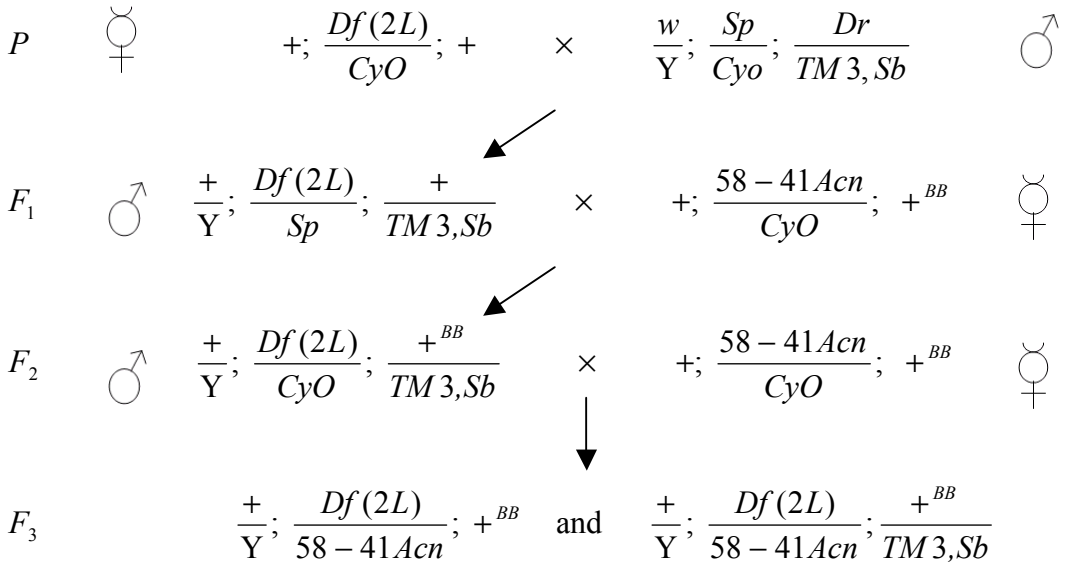


FIGURE 11.— Cross scheme for deficiency re-test in Experimental Strategy 3. In order to ensure the sterility mutation was not responsible for the male-male courtship, we initiated the cross scheme above. This approach enables the deficiency to be tested because the genetic background of the males tested is unambiguous and free of modifiers. Virgin males from the F_3 generation will be collected, isolated, and placed together in the same manner as with the BB assays (see text). If courtship is observed, then genes in the region will be analyzed one by one. If no courtship is observed, then a mapping strategy applied to the entire second chromosome will be applied.

If the results of the strategy are positive, then we will first test any genes in the region known to affect behavior. Genes in the span of the deletion can be individually researched to determine if any known mutant alleles have previously been determined to

cause male-male courtship. If not, then stocks with P-element insertions or other forms of gene knockouts can be ordered for each gene, enabling tests to be done one at a time to establish which gene is responsible for the abnormal behavior. Once a candidate gene is found, conduction of a complementation test will determine if our mutation is in the candidate gene; if male-male courtship is observed, then the candidate gene is likely to be the cause. Once a gene is found to be responsible, future endeavors include completely knocking down the gene followed by placing a good copy into another location in the genome. This test would determine if the normal phenotype could be "rescued," further proving the mutant gene's responsibility in producing the male-male courtship. Analysis into the molecular function of the gene can then begin after it has been shown to be required for normal male behavior.

In summary, we found the third chromosome was affecting the behavioral phenotype of our original mutants. Isogenization of the line resulted in stable male-male courtship observations that provided evidence of a mutation located on the second chromosome. Results from re-tests with deficiency chromosomes originally thought to not uncover the behavioral mutation will be able to tell us the next step in mapping the mutation causing male-male courtship.

CHAPTER 3

CONCLUSION AND HYPOTHESIS

Conclusion

As discussed by Anholt (ANHOLT 2004), neurological control of behavior cannot be completely dissected one gene at a time; the idea of networks of regulatory genes has quickly become apparent with the rise of the genomic era and must be studied accordingly. The single-gene approach is no longer sufficient in unraveling the complex control of biological processes such as behavior. Genes and their products can auto-regulate themselves along with influencing another gene's expression, splicing, translation, tagging, transport, and function, just to name a few. Of course, these impacts aren't necessarily acting on one aspect of phenotype; a disruption in a gene responsible for pigment distribution could potentially affect another involved in wing formation, resulting in a pleiotropic phenotype. It is truly a web of interactions in the sense that when one element is disrupted, the fidelity of the netting with which it interacts is impacted. Such was the case in this project: the function of a modifier on an entirely different chromosome acting to affect behavior was important to realize when preparing a strategy to pursue the unknown mutation. Though my hypothesis as to what may be happening is outlined below, it is important to keep in mind that because an idea sounds plausible doesn't mean that it is or can be fact—rigorous testing and support for that hypothesis are essential.

Hypothesis: Putting Names to Faces

Though *fruitless* has been accepted as the main regulator for behavior, I have identified a region on the second chromosome which can affect the behavior of males. Is this something acting downstream of *fru* or part of an entirely new branch in the *Sxi* pathway? Or, could it be serving as a link between *dsx* and the neurological control of behavior? In any case, one thing must be kept in mind when making a hypothesis: the EMS-generated mutation may not have completely inactivated the gene but instead could have slightly hindered its role in the cell. The thought of reduced function versus lack thereof widens the realm of possibilities.

I hypothesize that the original 58-41A *cn* stock contained at least two alleles of *fru* in the population. All of these allelic forms of Fru^M would be functional with the exception of binding affinities with cofactors. The disrupted gene (gene X) may encode for one of these cofactors and thus certain isoforms of Fru^M may not bind to it properly due to its deviation from the normal product. In other words, one form of Fru^M may be able to barely bind to the aberrant gene X product in spite of the mutation (thus a more normal phenotype is observed), while another form of Fru^M is not able to recruit the deformed gene X product (resulting in the abnormal male-male courtship). Though all variants of Fru^M may be functional in their normal cellular duties, the mutation in gene X is just enough to ensure its exclusion from interaction with certain allelic forms of Fru^M resulting in blocked transcription (Figure 12). Due to the inactivity of these target genes, a part of the male fly's nervous system is irregular, enabling it to court other males.

What aspects of the fly the target genes help produce are unknown, but it could be anything from the olfactory receptors to the pheromone production to the developmental innervation of neurons interpreting cues received during courtship. After the gene has been successfully mapped, further molecular tests can begin to unravel its function in the fly.

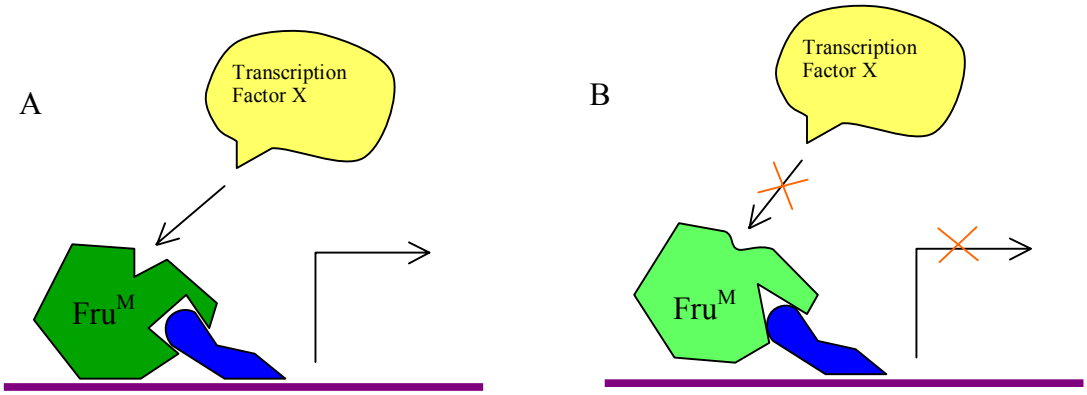


FIGURE 12.—Molecular model of the cause of male-male courtship. If two alleles of *fru* enabled two isoforms of Fru^M to be available in the cell, then perhaps one of them (dark green, A) is able to bind to the mutant protein (blue) but the other (light green, B) is not. In (A), there is enough affinity for the mutant protein to induce a conformational change in Fru^M, allowing Transcription Factor X to fully activate the target gene resulting in no male-male courtship. In (B), lack of sufficient binding to the mutant protein blocks the other transcription factors required for expression and the target gene remains inactive, resulting in the observation of a mutant male-male courtship phenotype.

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